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Conditioning for Conservation

A New Approach to Detection of Luteinizing Hormone in a Bottlenose Dolphin (*Tursiops truncatus*)

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Abstract

Precise determination of the time of ovulation will greatly enhance the success rate of artificial insemination and timed breedings of captive marine mammals. Because daily samples are necessary to effectively monitor the estrous cycle, behavioral conditioning of blood and urine collection has greatly facilitated the development of reproductive hormone profiles of the bottlenose dolphin (*Tursiops truncatus*). Measuring luteinizing hormone (LH) in urine obviates the need for blood collection and increases the frequency with which samples can be obtained. This non-invasive method has the potential to elucidate the temporal associations between the fall of circulating estrogen, the surge of LH, and the time of ovulation. Daily urine samples were collected from one female bottlenose dolphin during an ovulatory estrous cycle. Urine was concentrated and normalized by osmolality before application to an immunochromatographic assay (ICG) designed to detect canine serum LH. Centrifuging urine samples to remove insoluble components enhanced the definition and visibility of LH assay bands, concentrating urine increased the speed and intensity of test band development, and normalization of samples by osmolality ensured the application of a standard concentration of urine to each assay. In this single animal cycle follow-up trial, the urinary LH profile corresponded with serum LH, estrogen, and progesterone profiles, demonstrating the possible efficacy of this method for monitoring LH.

Key Words: bottlenose dolphin, *Tursiops truncatus,* luteinizing hormone, reproduction, endocrinology, urine, cetacean, operant conditioning

Introduction

The estrous cycles of marine mammals have been monitored by serum hormone levels, urinary steroid metabolites, urinary gonadotropins, and milk progesterone levels (West et al., 2000). In addition, follicle growth and ovulation have been documented with real time ultrasonography (Robeck et al., 1998, 2005; Brook, 2001). The duration of the estrous cycle of the bottlenose dolphin is estimated at approximately 30 d. Sawyer-Steffan et al. (1983) and Yoshioka et al. (1986) showed that female bottlenose dolphins may ovulate up to seven times per year or remain in anestrous for up to one year. While it has been reported that ovulation occurs 38 h after a luteinizing hormone (LH) surge in the killer whale (*Orcinus orca)* (Robeck et al., 2004), little is known about the relationship between the periovulatory fall of estrogen, the LH surge, and the time of ovulation in the bottlenose dolphin. Because the ovulatory LH peak is transitory, frequent hormone analysis is required to capture the peak. Schneyer et al. (1985) used a commercial radioimmunoassay (RIA) kit for human LH to measure serum LH in the bottlenose dolphin. As a non-invasive alternative to serum hormone analysis, urinary LH has been measured by RIA in the bottlenose dolphin (Robeck et al., 2005) and the killer whale (Robeck et al., 1993). This study on a single animal introduces a rapid method of monitoring LH levels in bottlenose dolphin urine using a commercial immunochromatographic assay (ICG) designed to detect serum LH in the domestic dog (*Canis familiaris*). Although there are no known reports of the assay's use with dog urine, its canine LH antibody reacts with serum LH from the sun bear (*Helarctos malayanus*) as well as serum and urinary LH from the giant panda (*Ailuropoda melanoleuca*) (Durrant et al., 2006). Because the structure of LH is highly

conserved among mammalian species (Liao et al., 2003), it was not unreasonable to expect that dolphin LH might also cross-react with the canine LH antibody. The objective of the current study was to determine the reliability of urinary LH detection in a female bottlenose dolphin using canine serum LH ICG assay kits. A well-documented ovulatory estrous cycle of one female bottlenose dolphin validated urinary LH assay results with serum LH, estrogen and progesterone profiles, and ultrasonography.

Materials and Methods

Animal Management

The 10-y-old nulliparous female bottlenose dolphin was housed with another female bottlenose dolphin in a 3,141,892-L saltwater pool, maintained at 21 to 23° C year-round at the Mirage Hotel in Las Vegas, Nevada. Approximately, 0.5 to 1.4 kg of fish were fed per day or a daily total of 7.3 kg (2.3 kg herring, 2.7 kg capelin, 1.4 kg surf smelt, 0.5 kg night smelt, and 0.5 kg of squid). Three multivitamin and mineral supplements formulated for marine mammals (Sea Tabs, Pacific Research Laboratories Inc, El Cajon, CA, USA) were incorporated into the daily diet.

Behavioral Conditioning

Operant conditioning techniques have proven to work well when training captive marine mammals (Ramirez, 1999). During operant conditioning, the consequences of a behavior influence the future frequency of the behavior. Positive primary reinforcers such as food are effective for strengthening a desired behavior (Baldwin & Baldwin, 1998). This method was the basis for training the dolphin for husbandry and research applications such as ultrasound exams, and blood and urine sampling. Slow, positive approximations were made for each behavior until the dolphin was fully conditioned for daily sampling and exams.

Ultrasonography Conditioning—The dolphin was trained to float laterally and stationary at the pool edge, supported by the submerged feet and legs of the ultrasonographer. The blowhole was submerged, but the animal could lift her head and take a breath as needed. Next, the unpowered ultrasound probe was gently touched to her skin and then moved along her body. Once she was comfortable with this procedure, the ultrasound machine was turned on, and the process was repeated. The duration of the exams increased as the animal's comfort level and behavior strengthened.

Blood Sampling Conditioning—The dolphin was trained to remain stationary, ventral side up, with her tail flukes resting on the legs of the trainer. Next, the dolphin was conditioned to allow a sterile preparation of the area from which blood was to be drawn. Following the skin preparation with isopropyl alcohol-soaked gauze, the dolphin was conditioned to remain calm as a needle was inserted into the vein and blood was drawn.

Urine Sampling Conditioning—The first step for this behavior was to place the dolphin in an ideal position for the sterile collection of urine. The position required the dolphin's genital slit and urethra to be sufficiently out of the water to assure an uncontaminated collection. First, the trainer stood on a dry ledge and grasped the tail flukes of the dolphin that was stationary in a dorsal position floating at the pool surface. The dry ledge was 7 to 10 cm higher than the water surface. Next, the trainer gently stepped backward pulling the dolphin's flukes backward and laterally over the ledge until the dolphin's genital slit was out of the water. The dolphin's head and pectoral flippers remained in the water. The dolphin's flukes were placed onto the ledge so that the genital slit was lateral and out of the water. Once the animal was comfortable with this position, it was necessary to condition the dolphin to urinate on cue. To capture the urination behavior, the trainer placed firm hand pressure to the dolphin's bladder, which often resulted in reflexive urination from the dolphin. Primary reinforcement was given to increase the frequency of this reflexive behavior. Over time, the dolphin was conditioned to urinate by simply placing her in the correct partially dry position, preparing the genital slit area (as described above in the "Blood Sampling Conditioning" section), placing a 50-ml sterile urine cup under the urethra, and gently touching the bladder region.

Ultrasonography

Ovarian follicles and corpora lutea were visualized with an SSD 900 Aloka ultrasound machine equipped with a 3.5 MHz transducer. Ultrasound imaging was performed each morning with the exception of 10 and 11 August. The ultrasound session typically required 3 to 5 min in both the left and right lateral positions. No coupling gel was required due to the lack of an air layer between the skin surface and the transducer. Images were recorded with a thermal printer.

Blood Collection and Hormone Assays

Blood was collected for 15 consecutive days (30 July through 14 August) for the evaluation of reproductive hormones. An average of 4 ml of blood was collected from a superficial fluke vein using a 23 to 25 gauge needle. Approximately half of the blood collected was placed in a plain red-top tube and refrigerated at 7.2° C for commercial RIA analyses of progesterone (P4, Antech Diagnostics, Irvine, CA, USA) and estradiol (E2,

Clinical Endocrinology Laboratory, University of California–Davis, Davis, CA, USA). The remaining blood was allowed to coagulate for 20 min in a 9-ml serum separator tube (Tiger Top Tube, Becton Dickinson, Franklin Lakes, NJ, USA) prior to centrifugation at 1,290 g for 5 min. An aliquot of fresh serum was applied to the LH assay according to the manufacturer's instructions for canine serum (approximately 100 µl). The remaining serum was stored in sterile polystyrene tubes at -33.9°C.

Urine Collection and Hormone Assays

The dolphin was trained to urinate on cue, and urine was collected in a sterile plastic specimen cup. An average of 5 ml of urine was collected daily for 50 d (23 July through 10 September) and immediately frozen at -33.9° C. Estradiol and progesterone were measured in dolphin urine using the electrochemi-luminescence immunoassay (ECLIA) tested with the Elecsys 2010 instrument (Roche Diagnostics, Mannheim, Germany) at Clinical Pathology Laboratory in Las Vegas (4275 S. Burnham Ave, Suite 325, Las Vegas, NV 89119, USA).

ICG Status-LH™ Assay

The ICG assay (LH assay, Canine Ovulation Timing ICG Status-LH™, Synbiotics, San Diego, CA, USA) is a semi-quantitative assay that produces a visible red test band in the presence of LH in canine serum. Color development of the control band indicates that the sample has sufficiently migrated across the assay test strip. A test band of equal or greater intensity than the control band indicates an LH value greater than 1 ng/ml. If no test band appears, or if the test band is less intense than the control band, the LH value is less than 1 ng/ml. Dolphin serum was prepared according to the assay kit manufacturer's directions and applied to the LH assay on each day of blood collection. Dolphin urine was thawed and applied to LH assays either neat (untreated) or after centrifuging, concentrating, and/or normalizing by osmolality. Results were scored and photographed at least 1 h after sample application.

Test band intensities were scored as follows (Figure 1):

0 = no visible test band
1 = faint test band
2 = test band less intense than control band
3 = test band and control band are equally intense
4 = test band more intense than control band

Experiment 1

Frozen urine samples were allowed to thaw at room temperature and then vortexed for 5 s. A 500 µl volume of urine from 10 August was applied to the LH assay either neat or after centrifugation (cUr) at 9,200 g for 3 min to remove cellular debris and other insoluble contaminants.

Experiment 2

A 100 µl volume of neat urine from 10 August was applied to the LH assay For comparison, urine was centrifuged and the supernatant was concentrated (ccUr) on a Microcon-10 filter device (Millipore Corp., Bedford, MA, USA) at 13,900 g for 35 min before application of 100 µl ccUr to the LH assay.

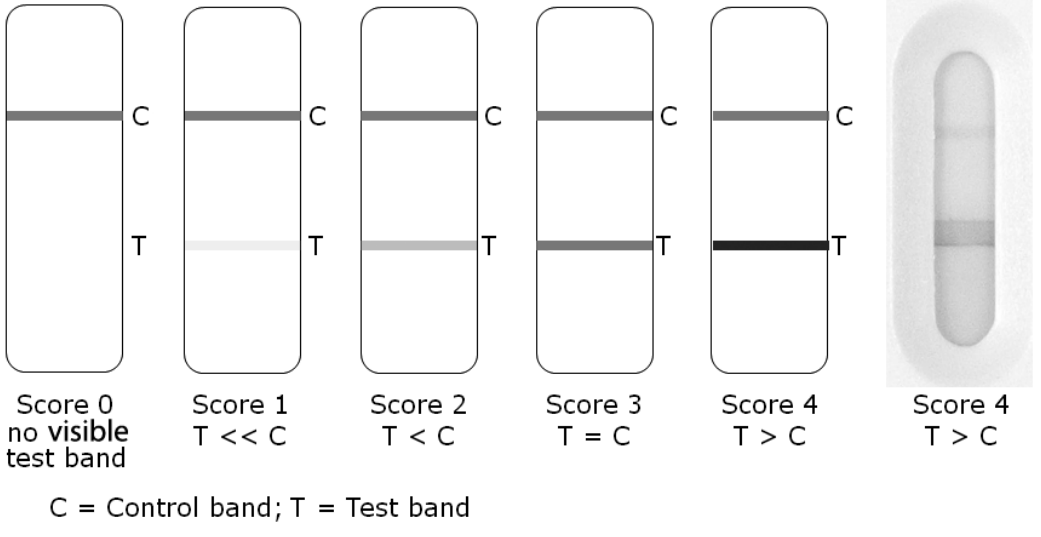


Figure 1. ICG LH assay scores

The filter device has a nominal molecular weight limit of 10,000 daltons, thereby enriching the urine retained on the filter for macromolecules including LH (30,000 Daltons).

Experiment 3

Osmolality is the ideal method for evaluating urine concentration as it depends equally on all analytes, regardless of molecular mass or structure (Dossin et al., 2003). To correct for initial differences in urine concentration, all samples were normalized by osmolality. A vapor pressure osmometer (Wescor Inc, Logan UT, USA) with a range of 0 to 1,999 mOsm/kg was calibrated with 290 mOsm/kg and 1,000 mOsm/kg standards. To obtain measurements within the range of the osmometer and to conserve the use of urine, 2 µl of each ccUr sample was diluted with 10 µl of sterile, distilled water prior to measurement of osmolality. For this study, each urine sample was concentrated and adjusted with sterile, distilled water (ccadUr) for a final application of 100 µL of 1,368 mOsm/kg per LH assay.

Results

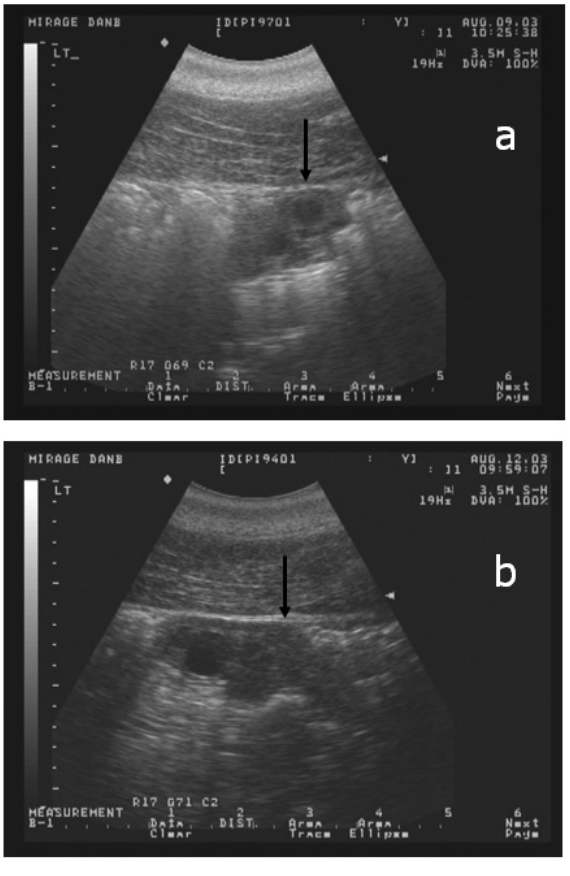
Ultrasonography

Ultrasonography confirmed the development of a dominant follicle measuring 1.9 cm on 9 August and a corpus luteum on 12 August (Figure 2).

Serum and Urinary Hormone Levels

Serum estradiol peaked on 9 August, and serum progesterone began to rise on 10 August (Figure 3). Serum LH, estrogen and progesterone values, and ultrasonography indicated that ovulation had occurred between 9 and 11 August. Dolphin serum collected on 10 August produced a positive LH ICG assay result.

Urinary metabolites of estrogen were elevated at the beginning of the test period on 1 August, peaking on 11 August. Progesterone metabolites began to rise following the fall of estrogen and remained high from 23 August to 4 September (Figure 4).



a. Left ovary on 9 August. Arrow: dominant follicle.

b. Left ovary on 12 August. Arrow: corpus luteum.

Figure 2. Bottlenose dolphin ovary ultrasound

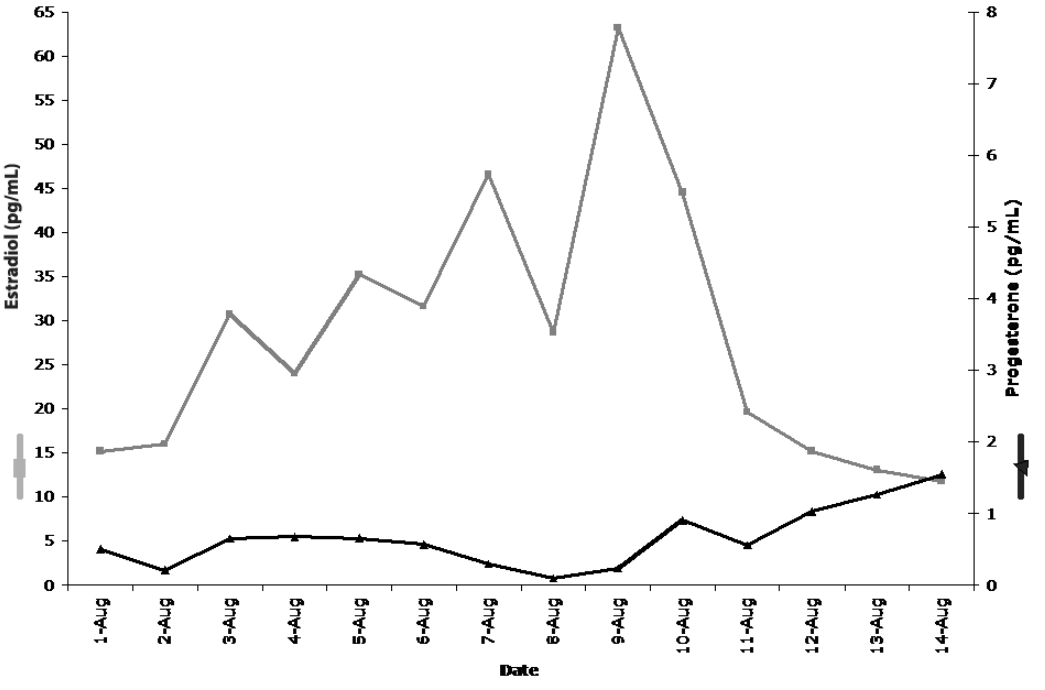


Figure 3. Serum estradiol and progesterone

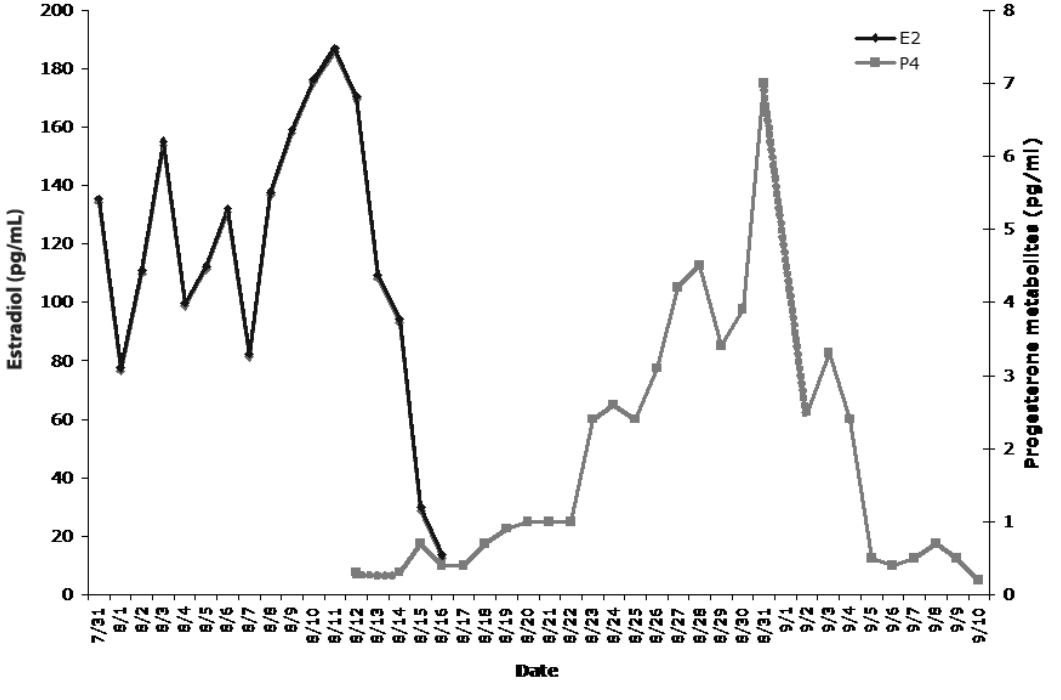


Figure 4. Urinary metabolites of estrogen and progesterone

Experiment 1
Although the recommended sample application volume is approximately 100 µl canine serum per LH assay, a larger volume of dolphin urine was applied to the assay in anticipation of a higher dilution of LH in urine compared to serum. A 500-µl volume of thawed, neat urine from 10 August produced a test band that was visibly more intense than the control band (score = 4), and 500 µl of thawed cUr produced a test band that was even greater in intensity relative to the control band (score = 4), along with a lower degree of nonspecific staining throughout the assay. Notably, after application of 500 µl of thawed urine, regardless of whether the urine was centrifuged or not, test band results were not visible until over 2 h after sample application. The normal recommended reading time of the assay by the manufacturer is 20 min when using canine serum.

Experiment 2
A 100-µl volume of thawed, neat dolphin urine from 10 August produced a test band on the LH assay that was visibly less intense than the control band (score = 2; Figure 5a). Centrifugation decreased or eliminated cellular debris from the urine and resulted in a test band equally as intense as the control band (score = 3; Figure 5b). After concentrating the urine nearly 5-fold, 100 µl ccUr produced a test band that was visibly more intense than the control band (score = 4; Figure 5c). When urine from 10 August was adjusted to a standard osmolality, the test band was less intense than the control band (score = 2; Figure 5d). After urine concentration (Figure 5c & d), the LH antibody reaction was rapid with visible results within minutes of sample application. LH assay results were scored 1 h after sample application, and control and test bands neither changed nor faded after months of storage at room temperature in the dark.

Experiment 3
Thawed and centrifuged urines from 1 to 15 August ranged from 1,232 mOsm/kg to 1,873 mOsm/kg. Significant pellets were visible after centrifugation of all urine samples. Normalization of samples by osmolality corrected for initial differences in urine concentration, ensuring the application of a standard concentration of urine to each assay. The ccadUr samples produced a urinary LH profile with a peak on 10 August and a pre-estrous LH surge on 1 to 4 August, which closely followed the serum LH profile (Figure 6). Urine volumes from 11 and 12 August were insufficient for the standard application of 100 µl ccadUr. An LH assay score of 0 was obtained from the application of 100 µl of 684 mOsm/kg urine from 12 August.

Discussion

This study of a single female bottlenose dolphin indicates that a simple method of monitoring urinary LH may facilitate detailed analyses of LH profiles during ovulatory estrous cycles and further clarify the relationship between LH, estrogen, progesterone, and ovulation in the dolphin. The ability

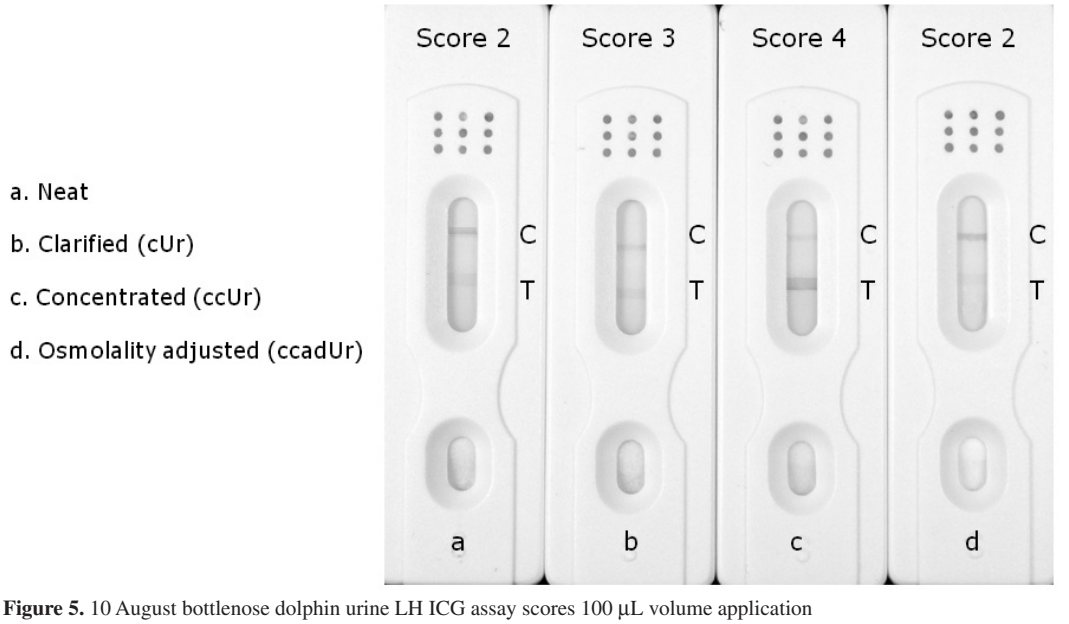


Figure 5. 10 August bottlenose dolphin urine LH ICG assay scores 100 µL volume application

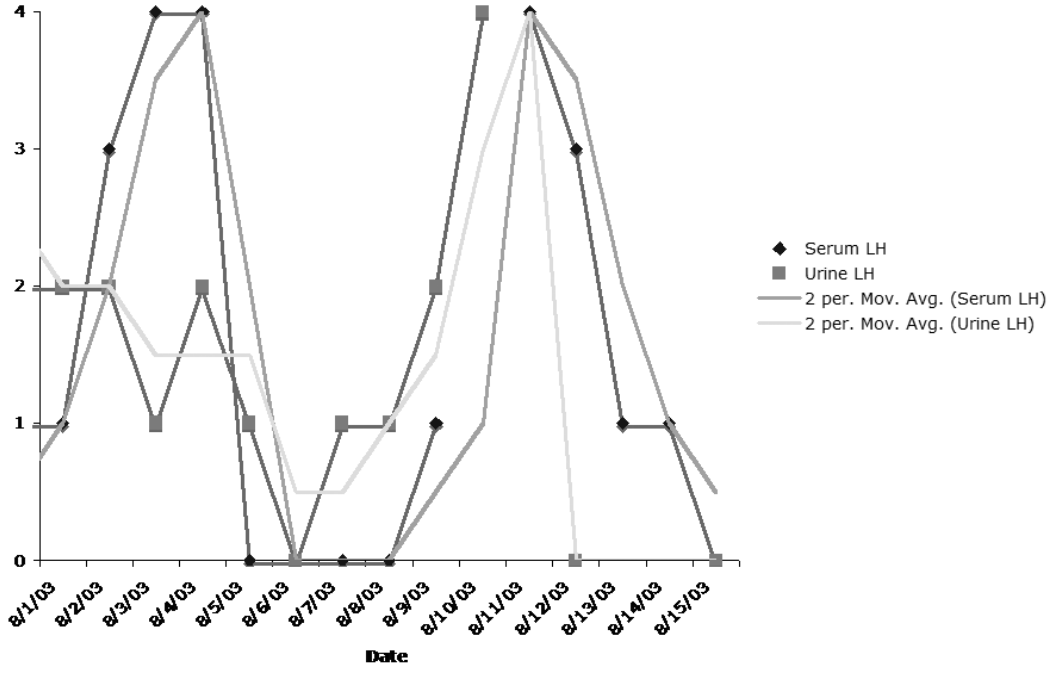


Figure 6. Serum and urine LH by ICG assay

to measure daily fluctuations in urinary LH is a valuable tool for the breeding management of captive populations. Although this method of urinary LH monitoring is semi-quantitative, it may be sufficient to distinguish pre-ovulatory LH from ovulatory LH levels. Furthermore, detection of a pre-estrous LH surge provides critical notification of impending ovulation, allowing animal care managers to prepare for artificial insemination procedures and/or pairing for natural mating. Conveniently, this method of urinary LH detection generates results within 1 h, much faster than conventional ELISA (enzyme-linked immunosorbent assay) or RIA methods of evaluating serum and/or urine. Monitoring LH in urine reduces the need to collect frequent blood samples to determine the time of ovulation. For a variety of captive species, blood collection can only be performed under anesthesia and can be stressful to the animal, whereas urine collection can often be a routine part of the animal care process. Marine mammal managers have successfully trained three captive cetacean species for urine collection behavior: the killer whale, the false killer whale (*Pseudorca crassidens*), and the bottlenose dolphin (Robeck et al., 1994). Additional bottlenose dolphin estrous cycles will be monitored to corroborate this study, and research in this laboratory will examine the utility of this novel detection method to evaluate reproductive status in the domestic dog and the giant panda.

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